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## Liquid-Liquid Phase Separation in Physiology and Pathophysiology of the Nervous System

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1    **Liquid-liquid phase separation in physiology and pathophysiology of nervous system**

2    Abbreviated title: LLPS in nervous system

3

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32 **ABSTRACT**

33 Molecules within cells are segregated into functional domains to form various organelles. While  
34 some of those organelles are delimited by lipid membranes demarcating their constituents, others  
35 lack a membrane enclosure. Recently, liquid-liquid phase separation (LLPS) revolutionized our  
36 view of how segregation of macromolecules can produce membraneless organelles. While the  
37 concept of LLPS has been well-studied in the areas of soft matter physics and polymer chemistry,  
38 its significance has only recently been recognized in the field of biology. It occurs typically between  
39 macromolecules that have multivalent interactions. Interestingly, these features are present in many  
40 molecules that exert key functions within neurons. In this review, we will cover recent topics of  
41 LLPS in different contexts of neuronal physiology and pathology.

42

## 43 INTRODUCTION

44 A neuron has a highly polarized and compartmentalized structure, which requires precise  
45 localization of various cellular components. Molecules synthesized in the cell body must travel long  
46 distances to reach their final destination. Upon reaching their destination, the molecules must be  
47 retained in an appropriate concentration relative to other factors. Additionally, the molecules may  
48 need to be segregated from their immediate environment, in order to establish a functional domain.  
49 Anomalies in this process can lead to pathological outcomes in the brain.

50         Compartmentalization of molecular processes is accomplished by various intracellular  
51 organelles that spatially segregate functionally related molecules. Major organelles such as the  
52 nucleus, endoplasmic reticulum, mitochondria, lysosome, endosome, etc. have demarcating  
53 membranes. In contrast, there are organelles that lack any demarcating membrane. These include  
54 the nucleoli, chromosomes, ribosomes, centrosomes, RNA granules, and stress granules. How such  
55 organelles maintain their constituent molecules was mostly overlooked in early studies using static  
56 images. However, a live-imaging study of P granules, cytosolic protein granules found in germline  
57 cells of *C. elegans*, revealed that these granules have liquid-like properties, including fusion, fission  
58 events, changes in size and reversibility (Brangwynne et al., 2009). At the same time, the molecules  
59 undergo constant exchange between the external environment, or dilute phase, and the condensed  
60 phase. This exchange was demonstrated by the photobleaching of fluorescently-labelled molecules  
61 (Brangwynne et al., 2009). These observations required us to re-think how membraneless organelles  
62 maintain their shape and constituents.

63         Subsequently, it was demonstrated that biological macromolecules including proteins and  
64 nucleic acids can condense and self-assemble into protein droplets *in vitro* (Kato et al., 2012; Li et  
65 al., 2012). Inside the condensate, the molecule can be enriched hundreds of folds compared with the  
66 original concentration in the cellular milieu (Zeng et al., 2018). In the simplest scenario, the  
67 molecules segregate from the solvent because they can exist more stably in a condensed phase than  
68 in a diluted phase, similar to the formation of oil droplets in a water-enriched environment. This  
69 phenomenon is called liquid-liquid phase separation (LLPS) because both diluted and condensed  
70 phases still retain properties as liquid (Hyman et al., 2014; Banani et al., 2017).

71         Importantly, the proteins condensed by the mechanism of LLPS still retain native  
72 physiological conformation and functions while undergoing exchange between the dilute and  
73 condensed phases. This is unlike more solid protein aggregates where the constituents proteins can  
74 be misfolded and immobile. However, LLPS can trigger the aggregation of proteins localized to the  
75 condensed phase (Hyman et al., 2014; Banani et al., 2017).

LLPS elucidates a wide variety of cellular functions, such as transcriptional and translational regulation, metabolism and catabolism, signal transduction, and cellular motility. It is possible that many reported protein-protein interactions mediating these cellular functions are actually part of a larger protein interaction network underlying LLPS. In this review we will discuss the role of LLPS in neurons, with a focus on local protein synthesis, synaptic organization, and neurodegenerative disease.

### *Biophysics behind LLPS*

LLPS has been well-studied in the field of soft-matter physics, but biologists have only recently discovered its importance and implications in divergent cellular functions (Hyman et al., 2014; Banani et al., 2017). The governing mechanism for forming phase-separated condensates in biological systems is multivalent interactions (Li et al., 2012; Banani et al., 2017; Chen et al., 2020). Such interactions can occur between molecules with multiple pairs of specific interactions (e.g. between multidomain scaffold proteins and their binding partners). An increase in multivalency lowers the critical protein concentration required for phase separation (Li et al., 2012). Multivalent interactions can also occur among proteins with intrinsically disordered regions, a region of protein without any fixed conformation or domain structure, or with various RNA species. Intrinsically disordered regions are often composed of low-complexity amino acids that are rich in hydrophilic residues (serine, glutamine, glutamate, arginine, and lysine) and which can form electrostatic interactions. Aromatic residues, such as phenylalanine, tyrosine, and tryptophan are stacked upon each other to form  $\pi$  electron cloud ( $\pi$ - $\pi$  interaction) or interact with positively charged residues via cation- $\pi$  interactions. In contrast, aliphatic residues, such as valine, leucine, and isoleucine, are less frequently observed in low complexity domains. Both protein-domain interactions and electrostatic interactions in the intrinsically disordered region contribute to the formation of condensed molecular assemblies with specific and distinct biological functions via phase separation.

In a simple two molecule system such as a protein in water, the phase behavior of the solution can be characterized by the free energy diagram (Fig. 1A) and the corresponding phase diagram (Fig. 1B). Under conditions relevant to living cells, most proteins in water form a homogenous one-phase solution due to the tendency of the mixture to increase its entropy (Fig. 1C). However, upon self-interaction, the protein may undergo liquid-liquid phase separation leading to two distinct phases: a highly condensed phase and a dilute phase (Fig. 1C). In the two-phase mixture, there is no free energy difference between the condensed and the dilute phases. The diffusion chemical potential ( $\mu$ ) of the protein generated by the concentration gradient between the two phases is offset by the net free energy gain ( $\Delta\Delta G$ ) of increased binding between protein

110 molecules in the condensed phase due to its higher concentration (i.e.  $\mu = \Delta\Delta G$ ). Thus, the phase  
111 separated liquid solution is at a thermodynamic equilibrium. Nonetheless, protein molecules in the  
112 condensed phase can freely exchange with molecules in the dilute phase (Fig. 1D).

113 The free-energy state of a two component mixture at any specific condition within the phase  
114 separation zone (pale blue and blue regions in Fig. 1B, see the corresponding free energy states of  
115 the regions in Fig. 1A) dictates that the system will spontaneously reach to two local minima,  
116 corresponding to  $\Phi_d$  and  $\Phi_c$ . Depending on the free energy state, phase separation can occur via  
117 binodal nucleation (formation of condensed phase requiring a nucleation processes) or spinodal  
118 decomposition (rapid and spontaneous phase separation without nucleation) (Fig. 1E). In a  
119 membrane-sealed compartment, exchange of molecules within and outside of the compartment  
120 needs to go through the membrane bilayer and requires energy (Fig. 1F). Thus, membraneless  
121 organelles are radically different from membrane-based organelles.

122 Due to the complexity of interactions between biological macromolecules, more than two  
123 condensates of different composition can form at the same time in the same cellular compartment.  
124 They can form independently of each other (phase-to-phase) or one condensate can form inside of  
125 another condensate (phase-in-phase) (Kato, 2012; Quiroz et al., 2020; Hosokawa, in press). This  
126 might account for subdomains observed in some membraneless organelles such as core-shell  
127 architecture of nucleoli, stress granules, and P granules (Kato, 2012).

128 To observe LLPS *in vitro*, proteins of interest are purified, fluorescently labelled, mixed, and  
129 observed by diffusion interference contrast (DIC) microscopy or fluorescence microscopy (Fig. 1E).  
130 Photobleaching of a single fluorescent droplet or part of a fluorescent droplet enables measurements  
131 of protein movement within the droplet as well as protein in exchange with diluted phase (Feng et  
132 al., 2019). These studies enable researchers to understand how protein components regulate LLPS  
133 in vitro, however, it is important to reproduce in vitro studies in the living cell.

134

### 135 *LLPS and local protein synthesis*

136 Membraneless organelles control gene expression, from transcription in the nucleus to local  
137 protein synthesis in distal processes (Martin and Ephrussi, 2009; Hnisz et al., 2017; Langdon and  
138 Gladfelter, 2018). These organelles circumvent the need for active transport of macromolecules  
139 across a membrane, enabling rapid signal transduction. While many of the membraneless organelles  
140 involved in gene expression share the biophysical trait of LLPS, each organelle is distinct in its  
141 molecular composition and function. Here, we focus on neuronal mRNA-containing  
142 ribonucleoprotein (mRNP) granules.

143 Proteins and mRNAs within neuronal mRNP granules can be dendritically localized  
144 (Kiebler and Bassell, 2006), where their translation can be regulated at synapses (Knowles et al.,  
145 1996; Kohrmann et al., 1999; Krichevsky and Kosik, 2001; Mallardo et al., 2003; Kanai et al.,  
146 2004) (Fig. 2). Retrograde and anterograde transport of these granules are microtubule-dependent  
147 (Knowles et al., 1996; Kohrmann et al., 1999). The movement of mRNAs to specific distal sites is  
148 necessary for synaptic plasticity and the strengthening of neuronal connections, a critical  
149 component of cognitive processes such as long-term memory (Richter and Lorenz, 2002; Klann and  
150 Dever, 2004).

151 LLPS of components of neuronal mRNP granules plays essential roles in mRNA trafficking  
152 and local protein synthesis (Fig. 2). Work from the Kandel and Fioriti laboratories posits a link  
153 between LLPS of cytoplasmic polyadenylation element binding protein 3 (CPEB3) in trafficking  
154 dendrite-bound mRNAs that contain cytoplasmic polyadenylation elements (CPEs) (Ford et al.,  
155 2019). Indeed, neuronal mRNP granules concentrate a large amount of CPE-containing mRNAs,  
156 including CaMKII $\alpha$  (Huang et al., 2003; Martin, 2004). The CPEs promote cytoplasmic  
157 polyadenylation-induced translation of the mRNAs in response to synaptic stimulation, such as  
158 NMDA-dependent long-term potentiation (Gu et al., 1999; Huang et al., 2006; Fioriti et al., 2015).  
159 Kandel and Fioriti have shown that CPEB3 binds CPEs of dendrite-bound mRNAs, providing  
160 translational regulation that is necessary for memory persistence (Fioriti et al., 2015). Additionally,  
161 they found that CPEB3 undergoes LLPS when bound to its target mRNA and is SUMOylated (Ford  
162 et al., 2019), suggesting that LLPS plays a role in translation regulation. Indeed, CPEB3 leaves the  
163 membraneless Processing Body (P body) to join the distally-located polysome after chemically-  
164 induced long-term potentiation (Ford et al., 2019). This work identifies the movement of phase  
165 separated, translation-dependent components from a repressed state in neuronal mRNP granule-like  
166 P bodies (Barbee et al., 2006) to an active state at distal ribosomes, and suggests that P bodies are  
167 playing an essential role in this process (Cougot et al., 2008; Ford et al., 2019).

168 Fragile X Mental Retardation Protein (FMRP) is another well-characterized component of  
169 neuronal mRNP granules, largely studied for its role in the pathogenesis of fragile X syndrome, the  
170 most commonly inherited form of mental retardation (Jin and Warren, 2003). Disruption of FMRP  
171 results in altered neural morphology in the form of excessively long and thin filopodia-like spines  
172 and fewer mature spines (Nimchinsky et al., 2001). FMRP is localized to the synapse upon  
173 metabotropic glutamate receptor activation, where it functions to target dendritic mRNAs and  
174 regulates translation (Jin and Warren, 2003; Antar et al., 2004). FMRP represses mRNA translation  
175 both *in vivo* and *in vitro*, possibly by blocking ribosome elongation at the polysome (Zalfa et al.,  
176 2006) and/or by microRNA-FMRP interaction, which would repress translation via the RNA-

177 induced silencing complex (Zalfa et al., 2006). Experiments conducted in vitro using reticulocytes  
178 extracts and recombinant FMRP suggest that this translation repression likely occurs within the  
179 LLPS state, since FMRP-containing droplets can recruit translational repressors and microRNA  
180 (Tsang et al., 2019). However the same authors do not show direct evidence that only the phase  
181 separated state is capable of repressing translation in an intact cellular environment. Thus additional  
182 studies are necessary to clarify whether the ability to repress translation is an exclusive property of  
183 the condensed phase. Interestingly, FMRP LLPS is mediated by binding to its mRNA targets and  
184 by post translational modifications such as phosphorylation (Tsang et al., 2019). Tsang et al. predict  
185 that additional RNA-binding proteins involved in translational repression might undergo LLPS to  
186 function as translational repressors in neurons (Tsang et al., 2019).

187 mRNAs in neuronal mRNP granules can also drive LLPS and direct dendritic targeting of  
188 mRNP granules. RNA modifies the LLPS behavior of RNA-binding proteins (Maharana et al.,  
189 2018), and the post-transcriptional state of the RNA, such as secondary structure, also plays a role  
190 in changing LLPS behavior (Langdon and Gladfelter, 2018; Van Treeck and Parker, 2018).  
191 Recently, the Jaffery lab identified a facilitating role of methylation of adenosine at the nitrogen-6  
192 position (m6A) in LLPS *in vitro*, and linked the high abundance of m6A RNA to LLPS of specific  
193 membraneless organelles (Ries et al., 2019). Interestingly, transcripts critical for synaptic  
194 organization and function are highly modified with m6A and are translocated to synapse  
195 (Merkurjev et al., 2018). Like the disrupted neuromorphology seen with FMRP mutations  
196 (Nimchinsky et al., 2001; Tsang et al., 2019), reducing the levels of the protein “m6A reader”, a  
197 protein that interacts with m6A-modified mRNA, caused structural and functional deficits in  
198 hippocampal dendritic spines (Merkurjev et al., 2018).

199 Local translation also takes place in axons (Jung et al., 2012; Wong et al., 2017; Hafner et  
200 al., 2019). Similarly to the local protein synthesis in dendrites, RNA-binding proteins play a major  
201 role in regulating axonal local translation (Antar et al., 2004; Kiebler and Bassell, 2006). A co-  
202 culture system of *Aplysia* sensory presynaptic and motor postsynaptic neurons has been used for  
203 studies of axonal local translation. After stimulation to induce long-term facilitation, relevant  
204 mRNAs, such as sensorin, rapidly concentrate in the presynaptic terminus of sensory neurons  
205 (Lyles et al., 2006). Moreover, live-cell imaging of fluorescent translational reporters revealed  
206 accumulation of newly synthesized proteins in the presynaptic terminus (Wang et al., 2009),  
207 suggesting local translation occurs in the presynaptic terminus during long-term facilitation.

208 As they are transported along axons to growth cones or presynaptic structures, RNA-  
209 binding proteins and mRNAs form mRNP granules through LLPS. Translation is suppressed in  
210 these granules until they receive extracellular signals that initiate local translation. FMRP, together



with proteins such as fragile X-related (FXR) 1 and FXR2, forms FMRP-containing granules (FXGs) by LLPS which plays an important role in the translation control (Antar et al., 2006; Li et al., 2009; Till et al., 2011; Parvin et al., 2019; Tsang et al., 2019). These granules are often localized near synaptic vesicles (Christie et al., 2009), which may serve as platforms for local translation at presynaptic structures. The synaptic vesicle protein synapsin 1 condenses into liquid droplets and promotes clustering of synaptic vesicles at presynaptic terminals (Milovanovic et al., 2018). Because FXGs localize with synaptic vesicles, it is possible that FMRP suppresses local translation to maintain mRNAs and translational machinery at the synapsin/synaptic vesicles condensate. Once a signal to initiate translation for synapse formation or plasticity is received, FMRP is dephosphorylated and FXGs are dispersed to initiate translation. The surrounding phase environment (synapsin/synaptic vesicles condensate) may affect the process of forming/dispersing FXGs by LLPS. However, further studies at higher resolution are necessary to detect translating ribosomes and FXGs in presynaptic structures in response to extracellular signals .

In summary, a multitude of nuclear and cytoplasmic membraneless organelles play critical roles in gene expression and local protein synthesis. The dense nature of these organelles, with high concentrations of select protein and RNA components, allow for “packets of information” to be delivered directly to relevant active sites. This allows for the efficient, and spatially-dependent, production of transcription and translation products in the polarized neuron.

#### *LLPS at the synapse*

Synaptic proteins are continuously turning over (Kuriu et al., 2006; Sharma et al., 2006) and yet synapses can persist for weeks, months or even the lifetime of the animal (Grutzendler et al., 2002; Yang et al., 2009; Isshiki et al., 2014). This is fascinating considering the synapse is an organelle that is not enclosed by a plasma membrane. A presynaptic terminus shows specific accumulation of component proteins, which tether the synaptic vesicles at rest and, upon the influx of  $\text{Ca}^{2+}$ , fuse them with a specialized part of the presynaptic membrane called the active zone. Postsynaptic receptors are embedded in the plasma membrane, beneath which, various cellular components involved in signal transduction and regulation are enriched and comprise the postsynaptic density (PSD) (Sheng and Hoogenraad, 2007). These pre- and postsynaptic structures lack any demarcating membranes that prevent the diffusion of the component molecules into the cytoplasm. Indeed, synaptic proteins turn over at rates ranging from minutes to hours, yet the synapse still maintains its molecular and structural identity over days and weeks (Grutzendler et al.,

2002; Kuriu et al., 2006; Sharma et al., 2006; Yang et al., 2009; Isshiki et al., 2014). These properties of protein accumulation are consistent with the phenomenon of LLPS (Fig. 3).

Synapsin is a presynaptic protein that crosslinks synaptic vesicles and tethers them to the cytoskeleton within the resting presynaptic terminus. Upon  $\text{Ca}^{2+}$  entry, activated CaMKII phosphorylates synapsin. This reduces the interaction of synapsin with synaptic vesicles and the cytoskeleton, and facilitates the process of vesicular release. When purified, synapsin can undergo LLPS *in vitro* in a manner recapitulating its *in vivo* properties (Milovanovic et al., 2018). Synapsin condensates can capture liposomes and are dispersed by CaMKII phosphorylation (Milovanovic et al., 2018). From these observations, synapsin is proposed to cluster synaptic vesicles in the presynaptic terminus by a LLPS-mediated mechanism.

The clustering of membrane surface proteins can also be regulated by LLPS of proteins that bind to intracellular regions of membrane proteins.  $\text{Ca}^{2+}$  comes into the presynaptic terminus through voltage-gated  $\text{Ca}^{2+}$  channels at the active zone of the presynaptic membrane. The clustering of the voltage-gated  $\text{Ca}^{2+}$  channels is mediated by two active zone proteins, Rab3-interacting molecule (RIM) and RIM-Binding Protein (RIM-BP) that interact with voltage-gated  $\text{Ca}^{2+}$  channels. RIM has a proline-rich domain and a PDZ domain, which interact with three SH3 domains in RIM-BP and with the PDZ binding motif of the N-type voltage-gated  $\text{Ca}^{2+}$  channels, respectively (Wu et al., 2019; Wu, 2020). Through these multiple domain interactions, RIM, RIM-BP, and voltage-gated  $\text{Ca}^{2+}$  channels can phase separate and form clusters at the active zone (Wu et al., 2019). Wu et al. (2020) demonstrated that purified synaptic vesicles coat the surface of the RIM/RIM-BP condensates either in solution or tethered to membrane bilayers by the cytoplasmic tail of voltage-gated  $\text{Ca}^{2+}$  channels, forming a new type of interaction between a membrane organelle and membraneless organelle. The coating of synaptic vesicles on the surface of active zone condensates implies that the total number of synaptic vesicles tethered to each active zone is determined by its surface area (Schikorski and Stevens, 1997). Remarkably, when the synapsin/vesicle condensates mixed with the vesicle-coated RIM/RIM-BP condensates, the vesicle-coated RIM/RIM-BP condensates are encapsulated by synapsin/small unilamellar vesicle (SUV) condensates, forming two distinct SUVs pools reminiscent of the reserve and tethered synaptic vesicle pools existing in presynaptic boutons. Thus, the authors have reconstituted a presynaptic bouton-like structure containing vesicle-coated active zone with one side attached to the presynaptic membrane and the other side connected to the synapsin-clustered synaptic vesicle condensates.

Purified postsynaptic scaffolding proteins Shank and Homer self-assemble into macromolecular complexes when they are mixed together *in vitro*. Both Shank and Homer are multimeric proteins, and Homer has Enabled/Vasp Homology (EVH) domain that interacts with

Shank (Hayashi et al., 2009). Through this multimer-multimer interaction, the protein complex takes on a high-order meshwork structure and is the proposed underlying framework of the PSD at the excitatory synapse (Hayashi et al., 2009). Similarly, SynGAP, a postsynaptic Ras activating protein, is a trimeric protein with a PDZ binding motif (Zeng et al., 2016). PSD-95, a postsynaptic scaffolding protein, multimerizes *in vitro* (Hsueh and Sheng, 1999; Zeng et al., 2018). When purified SynGAP and PSD-95 are combined, they form a macromolecular complex. Interestingly, the resultant complex has droplet-like structures consistent with the properties of LLPS (Zeng et al., 2016). The properties of these droplets, such as spontaneous formation, constant exchange between condensed and diluted phase, and spontaneous fusion, are consistent with the idea that these droplets are formed by LLPS. The phase separation of the PSD-95 and SynGAP mixture also suggests that the dense PSD assemblies beneath but not enclosed by the postsynaptic plasma membranes are formed via LLPS. A mutant that abolishes LLPS *in vitro* significantly impaired the enrichment of these proteins in neurons (Hayashi et al., 2009; Zeng et al., 2016).

When additional components of the PSD, including the NMDA receptor (NMDAR) subunit GluN2B (which has a PDZ binding motif), GKAP (which bridges PSD-95 and Shank), Shank, and Homer were added to a PSD-95/SynGAP mixture, this resulted in LLPS at lower protein concentration, indicating a synergetic effect on the phase formation (Zeng et al., 2018; Wu, 2020; Chen et al., 2020). However, the contribution of each protein to phase separate is different. Removal of PSD-95 significantly reduced GluN2B but not Shank and Homer. In contrast, removal of Shank significantly reduced Homer but had less impact on PSD-95 and SynGAP. This suggests that some proteins serve as a “driver” for the formation of phase separation while others serve as a “client”. PSD-95 serves as a major driver of phase separation while GluN2B serves as a client. In contrast, Homer and Shank form an independent layer that does not serve as a driver or client for PSD-95/SynGAP/GluN2B. This is consistent with electron microscopic observations of the laminal structure of PSD (Valtschanoff and Weinberg, 2001), where PSD-95 and GluN2B are layered together immediately beneath the synaptic membrane, while Shank is in a deeper layer. GKAP is an interesting molecule in this structure: when it was removed, both PSD-95/SynGAP/GluN2B and Shank/Homer had significantly reduced phase formation. GKAP is situated between these two layers in the protein complex and may serve as an interface. Indeed, in native PSDs, GKAP is layered between PSD-95/GluN2B and Shank (Valtschanoff and Weinberg, 2001).

AMPA type glutamate receptors (AMPA) are another major receptor group of the excitatory synapse. They interact with a myriad of proteins that regulate the synthesis, function, and subcellular distribution of AMPAR. Major interactors include the Transmembrane AMPA Receptor-interacting Proteins (TARPs), which interact with the transmembrane domain of

311 AMPARs and determine receptor localization and function (Nicoll et al., 2006). A prototypical  
312 TARP, Stargazin, can interact with PSD-95 through a PDZ-binding motif, as well as through an  
313 arginine-rich motif (Zeng et al., 2019). Through such multivalent interactions, Stargazin undergoes  
314 LLPS with PSD-95. This is required for efficient incorporation of AMPAR into the synapse.

315 The induction of synaptic plasticity can persistently alter the amount of the AMPAR and  
316 various other proteins residing at the synapse (Bosch et al., 2014). Thus, an important and  
317 outstanding question is how neuronal activity modulates postsynaptic LLPS to trigger the delivery  
318 of synaptic proteins. The induction of long-term potentiation (LTP) induces a delivery of  
319 postsynaptic proteins in a specific order from the dendritic shaft. Actin and actin-related proteins  
320 are the first to arrive at the synapse, followed by AMPAR. PSD scaffolding proteins such as PSD-  
321 95 and Homer take longer to increase (~2 hours) after LTP induction, and require the synthesis of  
322 new protein (Bosch et al., 2014). In contrast, SynGAP, another PSD protein that inhibits Ras  
323 activity, dissociates quickly from the synapse upon phosphorylation by CaMKII (Araki et al., 2015).  
324 Furthermore, phosphorylation of Stargazin by CaMKII negatively affects LLPS (Zeng et al., 2019).  
325 Because activation of CaMKII transiently occurs after LTP induction (Lee et al., 2009), this might  
326 create a time-window for reorganization of the postsynaptic protein condensate.

327 Indeed, CaMKII has several properties that enable it to undergo LLPS. Once activated by  
328  $\text{Ca}^{2+}$ /calmodulin, CaMKII can form a persistent complex with substrate proteins including the  
329 intracellular carboxyl tail of the NMDA receptor subunit GluN2B, Rac guanine nucleotide  
330 exchange factor (RacGEF) Tiam1, GJD2/connexin 36, LRRC7/densin-180, and the L-type  $\text{Ca}^{2+}$   
331 channel. In addition, CaMKII has a rotationally symmetric dodecameric structure that can  
332 simultaneously interact with these proteins and cross link them. The ability of CaMKII to undergo  
333 LLPS was experimentally demonstrated by using purified CaMKII and other PSD proteins,  
334 including the scaffolding protein PSD-95, GluN2B, and Stargazin as a proxy of AMPAR itself.  
335 Notably, CaMKII undergoes phase separation with these proteins only in the presence of  $\text{Ca}^{2+}$  and  
336 after it undergoes LLPS, this state persists even after chelation of  $\text{Ca}^{2+}$ . This persistence of LLPS  
337 after  $\text{Ca}^{2+}$  chelation requires phosphorylation of threonine 286 (T286) of CaMKII, which has been  
338 shown to render CaMKII constitutively active. Therefore, one major role of CaMKII at the synapse  
339 may be to link different postsynaptic molecules through LLPS in a manner triggered by  $\text{Ca}^{2+}$   
340 (Hosokawa, in press).

341 In a related study, Cai et al. discovered that autoinhibited CaMKII $\alpha$  specifically binds to  
342 Shank3. In a reconstitution buffer containing no  $\text{Ca}^{2+}$ , mixing CaMKII $\alpha$  and Shank3 leads to phase  
343 separation of the mixture. Addition of  $\text{Ca}^{2+}$  induces GluN2B-mediated recruitment of active  
344 CaMKII $\alpha$  and formation of the GluN2B/PSD-95/CaMKII $\alpha$  condensates, which is autonomously

345 dispersed upon  $\text{Ca}^{2+}$  removal. Protein phosphatases control the  $\text{Ca}^{2+}$ -dependent shuttling of  
346 CaMKII $\alpha$  between the two PSD subcompartments (the upper layer composed of GluN2B/PSD-95  
347 and the lower layer composed of GKAP/Shank3/Homer). Activation of CaMKII $\alpha$  further enlarges  
348 the PSD assembly, mimicking activity-induced structural LTP in synapse. Therefore,  $\text{Ca}^{2+}$ -driven  
349 and phosphatase-checked shuttling of CaMKII $\alpha$  between distinct PSD nanodomains may underlie  
350 structural plasticity of PSD assemblies via LLPS (Cai et al., in press).

351 LLPS of CaMKII is also involved in the segregation of synaptic surface proteins. Glutamate  
352 receptor subtypes are organized into nanodomains at the synapse. In each hippocampal synapse,  
353 NMDAR forms one dominant nanodomain and several small domains, while AMPAR segregates  
354 into several nanodomains of similar size surrounding the NMDAR. In contrast, metabotropic  
355 glutamate receptors (mGluR) are more diffuse (Goncalves et al., 2020). Postsynaptic nanodomains  
356 connect to the presynaptic active zone via cell adhesion molecules, thereby forming trans-synaptic  
357 nanocolumns (Tang et al., 2016; Biederer et al., 2017; Scheefhals and MacGillavry, 2018). CaMKII  
358 preferentially interacts with the NMDAR subunit GluN2B rather than the AMPAR, represented by  
359 Stargazin. This leads to the formation of a phase-in-phase structure of AMPARs within the  
360 NMDAR-CaMKII phase. Further, the cell-adhesion molecule neuroligin segregates with the  
361 AMPAR and connects the presynaptic neurexin with the presynaptic release machinery. This  
362 mechanism may place AMPARs just beneath the transmitter release site, thereby optimizing the  
363 transmission efficacy and serving as a novel mechanism CaMKII-mediated synaptic plasticity.

364 In contrast to prominent PSD assemblies in excitatory synapses, inhibitory synapses do not  
365 contain obvious dense thickening underneath synaptic membranes. However, recent cryo-EM  
366 tomography studies reveal a sheet-like dense assembly (referred to as iPSD) with a thickness of ~5  
367 nm (Tao et al., 2018). A recent study has demonstrated that glycine or GABA $_A$  receptors, together  
368 with gephyrin, a key scaffold protein in inhibitory synapses, can undergo phase separation, forming  
369 iPSD condensates. The formation of the iPSD condensates can be regulated by phosphorylation of  
370 gephyrin or binding of target proteins to gephyrin (Bai et al., 2020). Thus, analogous to excitatory  
371 PSDs, iPSDs are likely formed by phase separation-mediated condensation of scaffold  
372 protein/neurotransmitter receptor complexes.

373  
374 *LLPS in neurodegenerative disease.*

375 Neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) are currently incurable  
376 and have no effective treatments. To identify potential treatments, it is paramount to understand the  
377 cellular and pathological basis of disease. One defining cellular feature of neurodegenerative

378 disease is the deposition of protein aggregates in affected brain regions. Protein aggregates in a  
379 given disease are formed by a specific protein, e.g. the microtubule-associated protein tau (MAPT) in  
380 AD and 50% of patients with frontotemporal degeneration (FTD) (Mackenzie and Neumann, 2016;  
381 Vogels et al., 2020),  $\alpha$ -synuclein in PD and Lewy body dementia (Luna and Luk, 2015; Zbinden et  
382 al., 2020); and TDP-43 in >95% of patients with amyotrophic lateral sclerosis (ALS) and in ~45%  
383 of patients with FTD (Mackenzie and Neumann, 2016; Taylor et al., 2016). MAPT,  $\alpha$ -synuclein,  
384 and TDP-43 have an inherent capacity to aggregate; they harbor disease-causing mutations and the  
385 anatomical burden of these protein aggregates correlate with symptomatic decline (Luna and Luk,  
386 2015; Mackenzie and Neumann, 2016; Taylor et al., 2016; Harrison and Shorter, 2017; Vogels et al.,  
387 2020; Zbinden et al., 2020). How protein aggregates correlate with disease is unclear, but it is  
388 emerging that LLPS may be involved. Here we will focus on the role of LLPS in ALS.

389 ALS is an incurable motor neuron disease that leads to paralysis and death within 2-5 years  
390 of symptomatic onset (Taylor et al., 2016). In >95% of ALS patients, TDP-43 forms  
391 phosphorylated protein aggregates in the cytoplasm of affected motor neurons (Arai et al., 2006;  
392 Neumann et al., 2006). Mutations in several ALS-linked genes have been identified, and these give  
393 rise to ~15% of ALS cases (Taylor et al., 2016). Many of the mutated genes, including TDP-43,  
394 FUS, and TIA1, are RNA-binding proteins that harbor a prion-like domain (Sreedharan et al., 2008;  
395 Kwiatkowski et al., 2009; Vance et al., 2009; Kim et al., 2013; Mackenzie et al., 2017). The prion-  
396 like domain is an intrinsically-disordered region that can promote protein aggregation and protein  
397 phase separation both in vitro and in the cell (Johnson et al., 2009; Sun et al., 2011; Han et al.,  
398 2012; Kato et al., 2012; Lin et al., 2015; Molliex et al., 2015; Murakami et al., 2015; Patel et al.,  
399 2015; Xiang et al., 2015; Conicella et al., 2016; Ryan et al., 2018; McGurk et al., 2018a; McGurk et  
400 al., 2018b; Murthy et al., 2019; Conicella et al., 2020), and it is often the site of disease-causing  
401 mutations (Sreedharan et al., 2008; Kwiatkowski et al., 2009; Vance et al., 2009; Kim et al., 2013;  
402 Mackenzie et al., 2017). Thus, LLPS is a focus in the underlying pathogenesis of ALS.

403 In ALS, neurons are under constitutive stress that can arise from misfolded proteins in the  
404 endoplasmic reticulum and mitochondrial dysfunction (Kiskinis et al., 2014; Montibeller and de  
405 Belleruche, 2018). As a survival mechanism during stress, the cell inhibits global protein translation  
406 by sequestering RNA-protein complexes involved in the pre-initiation of protein synthesis into  
407 stress granules (Ivanov et al., 2019; Jaud et al., 2020). TDP-43 and several of the RNA-binding  
408 proteins linked to ALS localize to stress granules (Bosco et al., 2010; Dewey et al., 2011;  
409 Mackenzie et al., 2017; Fernandes et al., 2018). The hypothesis that stress granules are linked to  
410 ALS is further supported by evidence that demonstrates that disease-causing mutations in the RNA-  
411 binding proteins linked to ALS alter LLPS in vitro and localization of the respective proteins to



stress granules (Lin et al., 2015; Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015; Conicella et al., 2016; Lee et al., 2016; Lin et al., 2016; Boeynaems et al., 2017; Dao et al., 2018; Wang et al., 2018; McGurk et al., 2018b), that downregulation of pathways that promote stress granule formation mitigate TDP-43-associated toxicity and/or aggregation in various cellular and animal models (Elden et al., 2010; Kim et al., 2014; Becker et al., 2017; Zhang et al., 2018; McGurk et al., 2018c; Duan et al., 2019; Fernandes et al., 2020), and that stress-granule resident proteins co-aggregate with ~30% of TDP-43 inclusions in human ALS tissue (Liu-Yesucevitz et al., 2010; Bentmann et al., 2012; McGurk et al., 2014).

An overarching hypothesis has been that stress-granule localization of TDP-43 seeds the protein aggregation observed in ALS. Stress granules and LLPS condensates are highly concentrated sources of protein, which is a biophysical property that promotes LLPS. Thus, by increasing local protein concentration, LLPS provides an environment that can promote phase transition events that lead to the formation of protein oligomers with solid-like characteristics (Kato et al., 2012; Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015; Guo et al., 2018). In *in vitro* experiments, solid protein oligomerization within protein condensates can also be promoted by increasing the time the proteins are in the protein droplet, by repeated forming and dissolving the protein droplets, and by introducing disease-associated mutations to the protein (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). In line with these *in vitro* data, cells exposed to chronic stress form stress granules and persistent TDP-43 aggregates (McGurk et al., 2018b; Gasset-Rosa et al., 2019; Fernandes et al., 2020), suggesting that chronic stress and/or stress-granule localization leads to disease-like aggregation of TDP-43. However, under short-term stress, stress granules inhibit the formation of disease-like aggregates of TDP-43 and promote the solubility and dissolution of the protein after the removal of stress (McGurk et al., 2018b; Chen and Cohen, 2019; Gasset-Rosa et al., 2019; Mann et al., 2019; Fernandes et al., 2020). Thus, under short-term stress the cell controls both the accumulation and dissolution of TDP-43 aggregates, but under continued stress and maintenance of a condensed phase, TDP-43 transitions into disease-like aggregates.

Elucidation of the LLPS-associated dynamics of membraneless organelles and disease-causing proteins may explain the pathology observed in ALS and other neurodegenerative diseases. However, whether protein aggregation causes dysfunction and clinical symptoms is unknown. Data from animal models suggest that targeting pathways that promote LLPS and stress granule biogenesis is therapeutic (Elden et al., 2010; Kim et al., 2014; Becker et al., 2017; Guo et al., 2018; Zhang et al., 2018; McGurk et al., 2018c; Duan et al., 2019; Fernandes et al., 2020). Thus, studying the mechanisms of LLPS is directing us towards pathways with therapeutic potential for incurable diseases such as ALS.

446

447 *Concluding remarks*

448         LLPS is emerging as a key biological phenomenon that mediates several aspects of the basic  
449 organization and proper functions of cells in general, and neurons, in particular. It will be  
450 interesting to see where the field of LLPS will take us in the next few years. We anticipate that  
451 combined the technological advancements in super-resolution microscopy and other imaging  
452 techniques we will be able to fill the gaps between *in vitro* studies and *in vivo* conditions. Further  
453 advancements in our understanding of this phenomenon will also allow us to design new therapeutic  
454 approaches against neurodegenerative diseases.

455



456 **Figure Legends**

457 **Figure 1: Phase separation illustrated by a simple two-component system.**

- 458 (A) Free energy diagram showing phase separation of a two-component system (e.g. a protein  
459 indicated by blue dots in water indicated by brown dots) under a certain condition. A uniformly  
460 mixed system can undergo phase separation by lowering the free energy to its minima, which  
461 results in a two-phase system: a dilute phase ( $\Phi_d$ , expressed as fraction volume for the dilute  
462 phase) and a condensed phase ( $\Phi_c$ , fraction volume for the condensed phase).
- 463 (B) Phase diagram of the two-component system constructed by plotting the free energy minima as  
464 a function of temperature. The blue curve indicates a sharp boundary (or the threshold  
465 concentration) of the system transitioning from a homogenous single-phase state to a two-phase  
466 state. Within the phase separation region, two modes of phase separation, binodal nucleation  
467 and spinodal decomposition, can occur.
- 468 (C) In a phase-separated two-component system, a thermodynamic equilibrium is reached (i.e.  $\Delta G_{d/c}$   
469  $=0$ ). A sharp gradient in the concentration of the blue molecule is established between the two  
470 phases.
- 471 (D) After phase separation, the components of the condensed phase and the diluted phase can freely  
472 exchange. However, there is no net flow of components between the two phases.
- 473 (E) An example of binodal nucleation-induced phase separation forming condensed spherical  
474 droplets (*left*) and an example of spinodal decomposition-induced phase separation forming  
475 worm-like condensed networks (*right*).
- 476 (F) In sharp contrast to membraneless condensates, spontaneous compartment fusion or materials  
477 exchange do not occur in membrane-separated organelles.

478

479 **Figure 2. RNA binding proteins involved in RNA stability (P-bodies), mRNA transport**

480 **(mRNA transport granules), translation, and stress granules (SG) formation.**

481 Under transient stress, protein-protein and RNA interactions form a dense SG core. Several RNA  
482 binding proteins can be recruited to SG cores and undergo liquid-liquid phase separation forming  
483 functional dynamic structures (physiological LLPS). Under conditions of transient stress, SGs are  
484 transiently formed but disassemble after the stress is gone. In case of prolonged stress, and after  
485 post-translational modifications like phosphorylation, proteins can become insoluble (pathological

486 LLPS). The same RNA binding proteins can participate in the formation of non-toxic hydrophobic  
487 aggregates and toxic cytoplasmic inclusions.

488

489 **Figure 3. Schematic diagram LLPS at synapses.**

490 Synapses contain various unique biological condensates, such as active zones and post-synaptic  
491 density (PSD). In a presynaptic bouton (represented in light blue), the reserve pool of synaptic  
492 vesicles (SV) can form molecular condensates via coacervating with the synapsin condensates. The  
493 docked pool of synaptic vesicles instead coat the surface of active zone condensates formed by  
494 proteins including RIM, RIM-BP and ELKS. In the postsynaptic neuron (represented in purple) and  
495 both in excitatory and inhibitory synapses, formation of PSD assemblies may also involve phase  
496 separation of synaptic scaffold proteins interacting with neurotransmitter receptors.

497

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